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Award Number: DAMD17-99-1-9070

TITLE: USF-1 As An Inhibitor of Mammary Gland Carcinogenesis

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REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20020913 065

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> September 2001	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Sep 00 - 31 Aug 01)	
<b>4. TITLE AND SUBTITLE</b> USF-1 As An Inhibitor of Mammary Gland Carcinogenesis			<b>5. FUNDING NUMBERS</b> DAMD17-99-1-9070	
<b>6. AUTHOR(S)</b> Darryl L. Hadsell, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Baylor College of Medicine Houston, Texas 77030  E-Mail: dhadsell@bcm.tmc.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> <p>The hypothesis tested in this proposal is that overexpression of USF in the mammary glands of transgenic mice will inhibit myc-dependent tumorigenesis. To test this hypothesis, a transgene was constructed to target the overexpression of FLAG-tagged USF-2 to the mammary glands of transgenic mice under the control of the mouse mammary tumor virus (mmTV) long terminal repeat. A total of eight lines of transgenic mice were generated. Of these, one line demonstrated expression of flag-tagged USF-2 in the lactating mammary gland at levels 12-fold over that of endogenous USF-2. Analysis of mammary gland development and lactation in these mmTV-USF-2 transgenic mice supports the conclusion that overexpression of USF-2 has minimal impact. To determine the impact of USF-2 on myc-dependent mammary tumorigenesis, progeny from genetic crosses of the mmTV-USF-2 mice with transgenic mice that overexpress either c-myc or Ha-ras are currently being generated. Preliminary evaluation of tumorigenesis in these mice suggests that USF-2 by itself is not oncogenic. Analysis of tumorigenesis in larger populations of these transgenic mice will be completed in the coming year and will establish if mmTV-USF-2 can block myc-dependent tumorigenesis.</p>				
<b>14. SUBJECT TERMS</b> Breast Cancer, myc, transgenic mice				<b>15. NUMBER OF PAGES</b> 8
				<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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## Introduction

Upstream stimulatory factor (USF) consists of two helix-loop-helix/zipper (bHLH/zip) proteins, USF-1 and -2, which are highly conserved among species and related to *c-myc* (1) transcription factors. Previously published cell culture studies with cancer cell models show that USF is both antiproliferative and can antagonize *c-myc* (2). The research described in this proposal addresses the idea that expression/activity of USF is a determining factor in tumor initiation and/or growth. This idea was to be explored by testing the hypothesis that targeted overexpression of USF-2 in the mammary glands of MMTV-*myc* transgenic mice will cause withdrawal from the cell cycle and differentiation thereby preventing tumors. The overall approach was to make and characterize transgenic mice that overexpress USF-2 under the control of the mouse mammary tumor virus long terminal repeat. Once in hand this new line of transgenic mice would be crossed with a previously described line of transgenic mice that overexpress *c-myc* in the mammary gland. A decrease in tumor frequency and/or an increase in tumor latency among mice that carry both MMTV-*myc* and MMTV-USF-2 as compare with those which just carry MMTV-*myc* would confirm the hypothesis.

## Body

The approved statement of work for this project described two specific tasks to be completed over a 36-month period. The first task was to determine the effect of mammary-specific USF-2 overexpression on mammary gland development and lactation. This was to be completed during months 1 through 24. The second task was to determine the ability of mammary-specific overexpression of USF-2 to prevent *myc*-induced mammary tumors. This task was to be completed during months 9 through 36.

During the first 12 months of the funding period, the transgene construct was made and injected into FVB mouse embryos. This resulted in eight independent lines of transgenic mice. Although expression of mmtv-LTR-derived transgenes has been reported in mammary tissue from virgin mice(3), screening of virgin females from each of mmtv-USF-2 lines failed to demonstrate detectable transgene expression. This result supported the conclusion that a determination of the effects of overexpressing USF-2 on development of the mammary gland in virgin mice would be impossible. Because transcriptional activity of the mmtv-LTR is known to be dramatically induced with lactation, the major focus of task 1 for months 12 through 24 of the funding period was to complete a screen for transgene expression in lactating mammary tissue from mmtv-USF-2 lines and to determine the impact of such expression on mammary gland development and lactation. To complete the screen, females from each of six of the eight mmtv-USF-2 lines were mated and allowed to complete a normal pregnancy. At sixteen days postpartum, mammary gland biopsies were collected from these lactating females and used for the preparation of protein extracts for western blotting. Western blotting of these extracts using an anti-flag (Fig 1A) antibody to detect the epitope tag revealed that two of the transgenic females analyzed expressed detectable protein. Analysis of these extracts by western blotting with an antibody to USF-2 (Fig 1B) demonstrated a dramatic increase in the abundance of USF-2 in an F1 female from line 2904. Comparison of the abundance of this line with nontransgenic females demonstrated an eight-fold elevation in the abundance of USF-2. This result suggested that females from line 2904 would be useful for studies on the effects of USF-2 overexpression on mammary gland

development and tumorigenesis thus allowing for the completion of tasks 1 and 2 of the originally proposed studies.

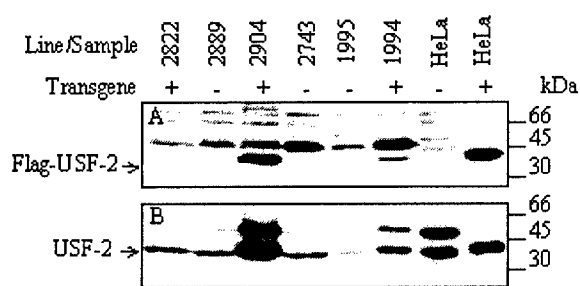


Figure 1. Screening of lactating mice for USF-2 transgene expression. Western blotting was used to detect the flag-tagged USF-2 transgene protein (A) or total USF-2 (B). Extracts were prepared from mammary tissue of nontransgenic (-) of transgenic (+) females at 16 days of lactation. Extracts from transfected HeLa cells were used as controls.

Because the transgene was only expressed during lactation, studies on virgin development in the mmtv-USF-2 transgenic mice were not conducted. To determine the effects of USF-2 overexpression on lactation, nontransgenic (n=4) and mmtv-USF-2 (n=4) mice were studied during the first eight days of lactation. The study was initiated by crossfostering the dams to litters of 10 pups each on day 2 postpartum. Comparison of average pup weight over the remaining 7 days of the study demonstrated a only a slight negative effect of USF-2 overexpression of pup growth. This effect was not statistically significant, however (P=0.14).

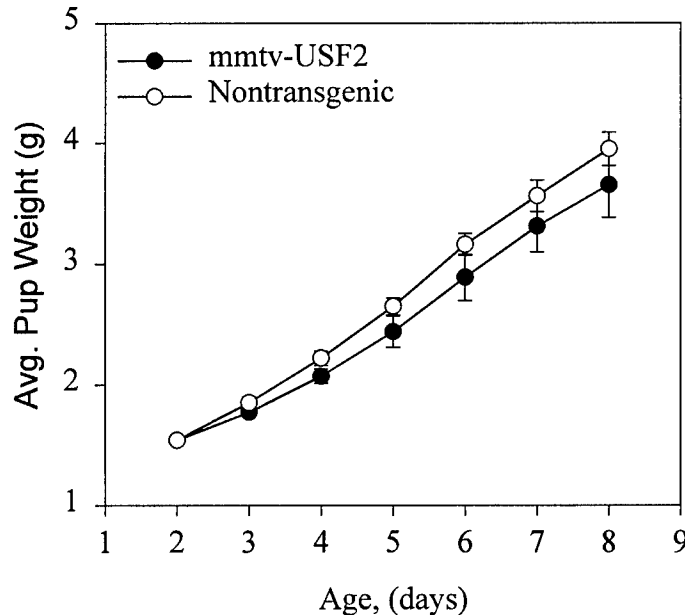


Figure 2. Growth of pups reared by nontransgenic or MMTV-USF-2 mice. On day 2 postpartum, nontransgenic (○) or mmtv-USF2 (●) dams were placed with crossfostered litters consisting of 10 pups each. Litterweights were collected daily for 8 days. On day 8, a milk sample was collected. On day 9 the dams were euthanized and mammary tissue was collected for biochemical analysis. Each symbol represents the mean±S.D. of repeated measures on four litters.

The effect of overexpression of USF-2 on mammary gland development during lactation was determined by comparison of morphology, wet-weight, and the content of DNA and protein within the mammary glands on day 9 postpartum. Comparison of

hematoxylin-eosin-stained sections demonstrated little difference between the morphology of nontransgenic and mmtv-USF-2 mice (data not shown). However, mammary gland wet weight was significantly reduced in the mmtv-USF-2 mice as compared to nontransgenic mice (Table 1). This reduction was not however accompanied by significant changes in the DNA or protein content of the mammary tissue.

Table 1 Analysis of mammary gland weigh, DNA and protein in mice that overexpress mmtv-USF-2 during lactation.

<i>Genotype</i>	<i>Wet weight</i>	<i>DNA, (mg/g)</i>	<i>Protein, (mg/g)</i>	<i>Protein:DNA</i>
Nontransgenic	0.51±0.09 <sup>a</sup>	4.6±1.6 <sup>a</sup>	108.8±28.3 <sup>a</sup>	25.0±10.9 <sup>a</sup>
mmtv-USF-2	0.38±0.02 <sup>b</sup>	4.3±0.6 <sup>a</sup>	102.5±9.2 <sup>a</sup>	25.6±7.4 <sup>a</sup>

<sup>ab</sup>Means within the same column with different superscripts differ ( $P < 0.05$ ). Each value represents the mean±S.D. for four animals.

To confirm the expression of the transgene western blotting was done for both flag and USF-2. Analysis of USF-2 abundance in mammary tissue extracts prepared at 9 days postpartum demonstrated an average elevation of 12-fold in the mmtv-USF-2 mice compared to nontransgenic mice (Fig 3A). To determine if overexpression of USF-2 affected mammary cell differentiation, milk protein abundance in mammary tissue extracts from these mice was analyzed by western blotting with an antibody specific for mouse milk proteins (Fig 3B). This antibody detects 7 different milk proteins, which can be distinguished by molecular weight. The most abundant of these is  $\beta$ -casein (fig 3B), which can be detected in as little as 10 ng of total lysate protein per lane. Little difference was observed for  $\beta$ -casein abundance among nontransgenic and mmtv-USF-2 mice supporting the suggestion that overexpression of USF-2 has minimal impact on mammary cell differentiation.

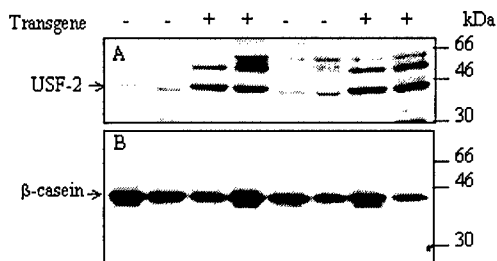


Fig 3. Analysis of USF-2 (A) and milk protein (B) abundance in lactating nontransgenic (-) and mmtv-USF-2 (+) mice. Mammary tissue extracts were prepared from 4 mice of each genotype on day 9 postpartum. For the USF-2 blot (A), 10 mg of protein were loaded per lane. For the milk protein blot, 10 ng of protein were loaded per lane. Equivalency of protein loading was confirmed in parallel coumassie-stained gels (not shown).

The completion of task 1 for the original statement of work has provided a line of transgenic mice with which to study the ability of overexpressed USF-2 to inhibit myc-dependent mammary tumorigenesis (task 2). Work on a modified version of task 2 commenced in December of 2000 and consisted of making genetic crosses between the mmtv-USF-2 mice and two other strains of transgenic mice; mmtv-myc (4) and mmtv-v-Ha-ras (5). The goal of these studies to generate 20 female mice for each of five different genotypes (Table 2). To date, almost half of the required number of animals have been obtained for the mmtv-USF-2 and mmtv-myc mice and about 30% of the USF-2/myc bigenics have been obtained. Only 2 myc/ras bigenics and no trigenic mice have thus far been obtained. Of the mice studied to date, half of the mmtv-myc mice have developed tumors, while only 16 % of the bigenic myc/USF-2 mice have developed tumors. No tumors have yet been observed in the mmtv-USF-2 mice. Although this data is preliminary and more animals will be added to the study during the next few months, it does support the conclusion that overexpressed USF-2 is not oncogenic.

Table 2. Current status of tumorigenesis study of transgenic mice that overexpress different combinations of USF-2, c-myc and v-Ha-ras.

<i>genotype</i>	<i>Mice obtained</i>	<i>Age, (days)</i>	<i>Lactations</i>	<i>Tumors</i>	<i>Latency, (days)</i>
mmTV-USF-2	7/20	123±3	3±1	0/7	--
mmTV-myc	8/20	137±16	3±2	4/8	150±12
USF-2/myc	6/20	91±8	1	1/6	103
myc/ras	2/20	131±0	1	0/2	--
USF-2/myc/ras	0/20	--	--	--	--

#### Key Research Accomplishments

- Screened six founder lines of transgenic mice for expression during lactation.
- Obtained one line of transgenic mice capable of 8-fold overexpression of USF-2 within the mammary gland during lactation.
- Determined the impact of overexpressing USF-2 on lactational capacity and the expression of milk proteins.
- Obtained transgenic (bigenic) mice carrying both mmtv-myc and mmtv-USF-2 transgenes.

#### **Reportable Outcomes**

- Development and preliminary characterization of a line of transgenic mice that expresses USF-2 in the mammary gland during lactation at levels 12 fold above endogenous USF-2.

- Obtained bigenic mice with which to evaluate the ability of USF-2 to block myc-dependent tumorigenesis.

## Conclusions

In summary, the data collected to date suggest that overexpression of USF-2 in the mammary gland has little, if any effect, on the overall development or the ability to lactate. The trend towards a lower growth in pups suckled on mmtv-USF-2 transgenic dams, coupled with the small, but significant reduction in mammary gland wet weight at day 9 postpartum, Supports the conclusion that if USF-2 overexpression does influence lactation, the effect is small and will require larger animal numbers for reliable detection. Preliminary data obtained from the tumorigenesis studies, supports the conclusion that a valid test of the ability of USF-2 to block myc-dependent tumorigenesis in these transgenic mouse models will be forthcoming. The remainder of the funding period will focus on further characterization of the effects of USF-2 overexpression on mammary gland development using the lactation model and on completing the Tumorigenesis studies by expanding the genetic crosses to obtain the required number of animals for each genotype.

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## Appendices

None